Protein protocols – Western blotting

A. Protein sample prep

1. Grow cells in LB and appropriate antibiotic
2. Harvest cells, pin down cells in sterile tubes or centrifuge bottles
3. Resuspend in 50 mM Tris-HCl, 2 mM EDTA, and 0.2 mM PMSF (keep samples on ice)
4. Lyse cells by sonication, freeze/thaw or chemical means –
    a. Sonication
        i. Clean sonicater tip 2-3 times with DI H_2O
        ii. Sonicate 2 to 3x for 30 sec – 1 min at 5 amplitude 5 (time varies depending on sample size). Make sure tip is immersed in cell solution and no foaming occurs when sonicating.
        iii. Keep samples on wet ice! Samples will heat up. Let cells rest between sonication cycles for 30 sec.
    b. Freeze/thaw
        i. Add lysis buffer and suspend cells
        ii. Put cells in -20°C freezer for 1+ hours to freeze.
        iii. Remove cells and thaw in ice (for multiple cycles) or at room temperature
        iv. Repeat steps ii - iii 2-3 times
5. Transfer cell lysates to a sterile 1.5 ml microcentrifuge tubes (or larger if needed)
6. Spin at 4°C and max rpm for 30 minutes to separate soluble and insoluble fractions
7. Transfer supernatant to clean 1.5 ml tube and label as soluble fraction (keep on ice or store in 4°C)
8. Optional: resuspend insoluble fraction in 0.5 - 1 ml 8 M urea (keep volume the same as soluble fraction). Incubate at 100°C for 5-10 min and recentrifuge 10 minutes at room temp and max rpm. Save supernatant as insoluble fraction. Store in 4°C

B. Bradford or DC protein assay (BioRad) – From Microplate protocol

1. Pipet 5 µl of prepared standards and samples into clean dry microtiter plate. Usually do samples undiluted and in a 1:5 or 1:10 dilution
2. Follow kit protocols for microplate

C. Protein Gel - BioRad gel system

1. Prepare Samples (~15-30 µl in a well depending on # of lanes) – dilute protein in buffer if needed and Laemmli sample buffer (1:1 volume). Make sure reducing agent has been added to Laemmli (add 56 mg of DTT to 950 µl sample buffer and adjust volume to 1 ml)
2. Heat samples at 95 - 100°C for 5-10 min
3. Warm up standards to room temperature (if running western, make sure to use western standard: Precision plus protein westernC standard from BioRad).
4. Set up protein gel – Use Mini TGX gels from BioRad (stored at 4°C)
    a. Open package (pour out any excess buffer) and remove gel cartridge
    b. Remove green strip on bottom of cassette to expose foot (needed to expose gel to buffer when running)
    c. Remove comb carefully (press straight up)
    d. Rinse wells with DI H_2O to remove residual buffer
    e. Place in Tetrad protein cell (see diagram in direction booklet)
    f. Fill inside compartment with **fresh** 1X Tris/glycine/SDS buffer to top , then outside to correct level (dependent of # of gels) with used buffer
    g. Fix any lanes that are deformed

5. Load gel with protein samples
6. Run @ 190V for 35 minutes. Dye should run until black line near bottom of cassette.
7. After run gel is removed by "cracking" casing around gel – wet fingers with DI water before handling gel
8. After running gel, put used buffer in used Tris/Glycine bottle. Clean cell with RO water and store dry. Never leave buffer in cell.

D. Western Blot – Transfer proteins to membrane
   1. Soak ImmunBlot PVDF Membrane (0.2μm) in MeOH for 30sec to 2 min
   2. Soak 2x extra thick blot paper (criterion size) in 1X Nupage transfer buffer
   3. Soak membrane in transfer buffer between filter paper
   4. Remove TransBlot SD semidry transfer cell (words facing you) and remove lid and inner top
   5. On transfer cell lay blot paper then PVDF membrane
   6. Carefully place gel on PVDF membrane with foot facing up and no bubbles! – be careful not to move around on membrane
   7. Cut off the raised foot of gel and place blot paper on top
   8. Use pipet to remove excess buffer by rolling over blot paper – don't move gel around!
   9. Check membrane to make sure it is still lined up
   10. Reassemble apparatus, Plug into machine
   11. run @ 15V for 30 minutes (proteins will travel down onto membrane) – be careful removing membrane at end.
   12. Dry surface, rinse with DI well, then 70% EtOH and let dry before storing

E. Western blot – Blotting
   1. Make blotting buffer – 50 mL 1X TBST w/ 2.5 g milk protein Buffer is used to coat surface of membrane with proteins
   2. Carefully remove gel from membrane. Make sure protein transfer occurred.
   3. Incubate in 50ml blocking buffer for at least 1 hr, continuous rocking
   4. Remove blocking buffer. Add 10ml blocking buffer to membrane.
   5. Add CaM primary antibody 1:5000 to 1:20000 dilution
   6. Incubate 2 hrs at room temperature, Optional: add more 1X TBST to prevent membrane from drying out and incubate overnight
   7. Quick wash 2x with 10 ml 1X TBST
   8. Wash 3x with 10 ml 1X TBST for 5 minutes (rocking)
   9. Incubate 1 hr in 50 ml 1X TBST with 1 μl secondary antibody (goat anti-rabbit)
   10. 2x quick wash with 25 ml 1X TBST
   11. 3x wash with 50 ml 1x TBST for 10-20 min
   12. Prepare imaging station for
   13. Mix Immun-Star westernC Chemiluminescent kit reagents – 1 ml of luminol/enhancer and 1ml peroxide buffer
   14. Remove wash buffer from membrane and add 2ml of kit reagents. Make sure there are no air bubbles so surface is completely covered with substrate
   15. Drain membrane by touching one corner of membrane to kimwipe and let solution wick off. Do not let it dry completely
   16. Develop using Bio-Rad molecular imager to analyze (see protocol there)